British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2011.01514.x
www.brjpharmacol.org

REVIEW

# Desmoteplase: discovery, insights and opportunities for ischaemic stroke

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**Keywords**
desmoteplase; tissue-type plasminogen activator; vampire bat; stroke neurotoxicity; thrombolysis

**Received**
28 February 2011
**Revised**
16 May 2011
**Accepted**
19 May 2011

Nature has provided a vast array of bioactive compounds that have been exploited for either diagnostic or therapeutic use. The field of thrombosis and haemostasis in particular has enjoyed much benefit from compounds derived from nature, notably from snakes and blood-feeding animals. Indeed, the likelihood that blood-feeding animals would harbour reagents with relevant pharmacology and with potential pharmaceutical benefit in haemostasis was not too far-fetched. Blood-feeding animals including leeches and ticks have evolved a means to keep blood from clotting or to at least maintain the liquid state, and some of these have been the subject of clinical development. A more recent example of this has been the saliva of the common vampire bat *Desmodus rotundus*, which has proven to harbour a veritable treasure trove of novel regulatory molecules. Among the bioactive compounds present is a fibrinolytic compound that was shown over 40 years ago to be a potent plasminogen activator. Studies of this vampire bat-derived plasminogen activator, more recently referred to as desmoteplase, revealed that this protease shared a number of structural and functional similarities to the human fibrinolytic protease, tissue-type plasminogen activator (t-PA) yet harboured critically important differences that have rendered this molecule attractive for clinical development for patients with ischaemic stroke.

### Abbreviations

BBB, blood–brain barrier; DIAS trial, Desmoteplase in Acute Stroke trial; DSPAα1, desmoteplase (also referred to as *Desmodus* salivary plasminogen activator); ICH, intracerebral haemorrhage; LDLR, low-density lipoprotein receptor; LRP-1, low-density lipoprotein-related receptor-1; NMDAR, NMDA receptor; PAI-1, plasminogen activator inhibitor type 1; PDGF-CC, platelet derived growth factor-CC; sct-PA, single-chain tissue-type plasminogen activator; t-PA, tissue-type plasminogen activator; tct-pA, two chain tissue-type plasminogen activator

## Introduction

Blood clotting is an essential phenomenon that is not only required to initiate the vascular repair processes but also provides the first line defence of the innate immune system. Activation of the blood coagulation cascade ultimately results in the cleavage of fibrinogen into fibrin, which polymerizes and forms the structural component of a clot to maintain clot rigidity and stability. The haemostatic system maintains a fine balance between clot formation and clot dissolution. Clots formed under normal conditions are necessarily short-lived and removed in a timely manner by another endogenous enzyme cascade referred to as the fibrinolytic system (Cesarman-Maus and Hajjar, 2005). The effector enzyme of this system is the serine protease, plasmin which is generated from its zymogenic precursor, plasminogen, by the plasminogen activators. There are two such plasminogen activators, both serine proteases, known as urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA).

The fibrinolytic system is conserved among all warm-blooded species studied to date. Curiously, some species have evolved unique features of their coagulation and fibrinolytic systems in part as a consequence of their lifestyle demands (Zavalova *et al*., 2002). Bats provide a remarkable example of this evolutionary process. To enable the feeding transition of bats from fruit and insects, to bird blood and later to mammalian blood, the saliva of these animals evolved a family of closely related plasminogen activators that have been

optimized phylogenetically for the removal of clots from the blood harvested during their feeding (Schleuning, 2001). However, in the mammalian system, plasminogen activation is not only needed for fibrinolysis. Indeed, as will be outlined in greater detail in later sections, plasmin generation in mammals is not always related to blood clot removal, particularly in the brain, while the plasminogen activators themselves also have proven to have functions independent of plasminogen. Hence, the human plasminogen-activating system evolved to accommodate these more diverse needs at the expense of being focused purely on fibrinolysis, which is a key characteristic of the fibrinolytic system in the vampire bat.

The fibrinolytic moiety in the saliva of the common vampire bat, *Desmodus rotundus*, selected for clinical development was originally described as 'desmokinase' (Hawkey, 1966) then '*Desmodus* salivary plasminogen activator (DSPA)' (Kratzschmar *et al*., 1991; Schleuning and Donner, 2003) and finally 'desmoteplase'. Desmoteplase is, to all intents and purposes, the vampire bat's orthologue of human t-PA. However, despite this similarity, head-to-head comparisons between t-PA and desmoteplase have revealed vastly different biochemical properties between these two proteases, which can for the most part be explained on structural grounds. This review will outline the history of desmoteplase, its biochemical and pharmacokinetic profile against t-PA and plasminogen activators from other species of vampire bats, its comparative effects in the brain and a short review on the status of clinical trials of desmoteplase in patients with ischaemic stroke.

## History of desmoteplase

There are approximately 1100 species of bats worldwide that constitute ~23% of all mammalian species on earth. Bats are therefore an immensely successful group of mammals, and the evolution of flight, ecolocation, olfaction and feeding diversity (insects, fruit. nectar and blood) have certainly been key elements in their success as a species.

Of these 1100 species of bats, only three species have adapted to blood feeding, a dietary trait referred to as haematophagy. These sanguinivorous bats include the common vampire bat (*D. rotundus*), the hairy-legged vampire bat (*D. ecaudata*) and the white-winged vampire bat (*D. youngi*). However, despite their common diet, these species of vampire bats prefer to diet on blood from different species, the biochemical basis for this preference is now becoming clearer.

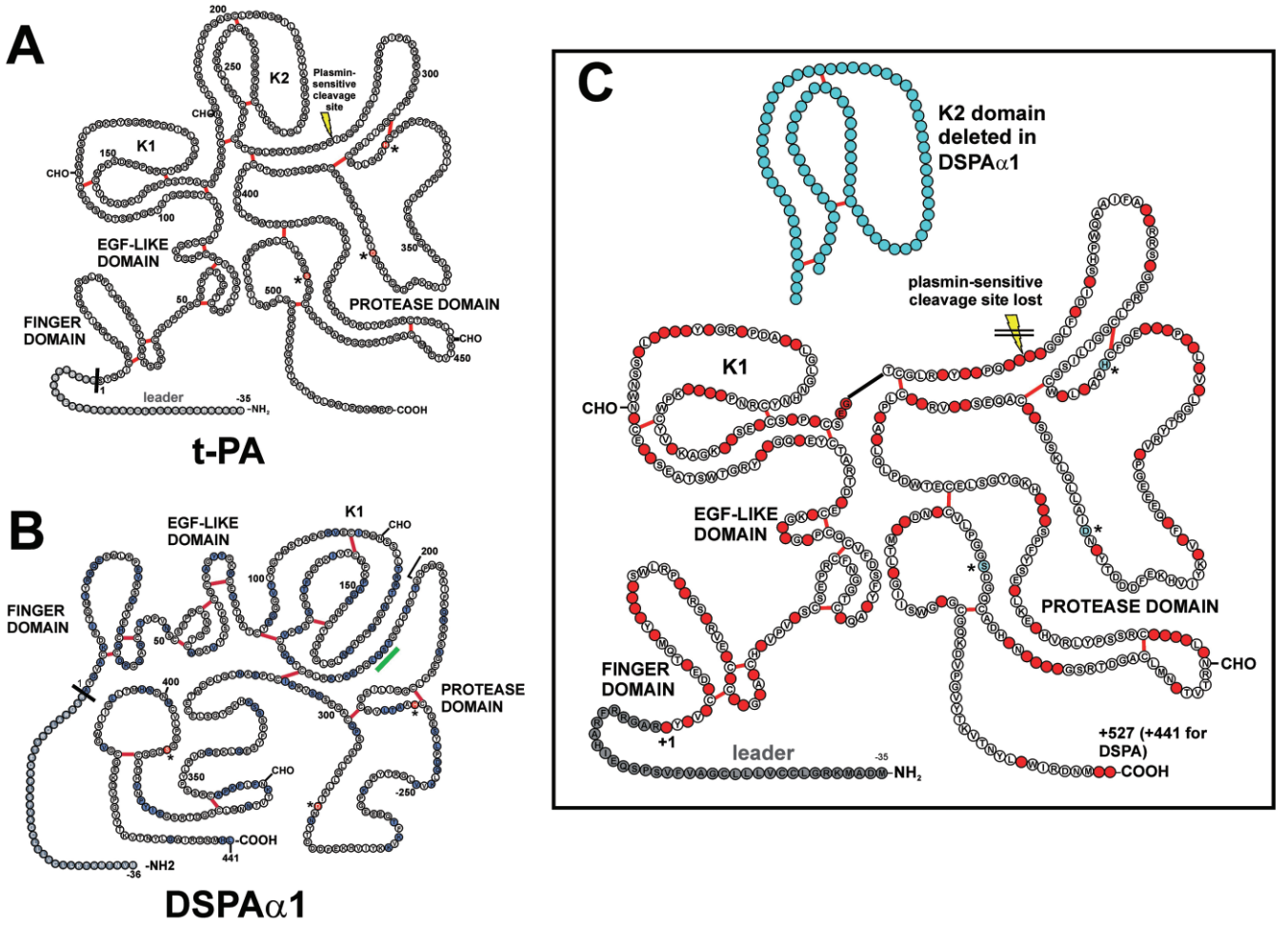
Due to the relevant abundance of *D. rotundus* compared with the other two species, the saliva of this particular species has been studied in greater detail. Over the years, the saliva of *D. rotundus* has been shown to contain a highly targeted fibrinolytic system enzyme, a potent platelet aggregation inhibitor (Hawkey, 1967) and a novel anti-coagulant (Apitz-Castro *et al*., 1995; Fernandez *et al*., 1999). A vasoactive compound has been detected but not characterized in any detail (Hawkey, 1966). Of these, the fibrinolytic agent in the saliva has been of particular interest.

The saliva of the common vampire bat *D. rotundus* was first shown to harbour a fibrinolytic principle in 1932 (Bier, 1932). That this agent was in fact a plasminogen activator was described in 1966 (Hawkey, 1966). In this study, *D. rotundus* saliva was shown to lyse fibrin plates in a plasminogen-dependent manner and to cause lysis of human plasma clots. It was later revealed that saliva-induced lysis only occurred in the presence of fibrin (Cartwright, 1974), a feature that would later prove to be critical for its future development. It took, however, another 15 years before this plasminogen activator was cloned and characterized and its enzymatic profile compared with the more widely known plasminogen activators. cDNA cloning of the vampire bat protease was reported by two groups (Gardell *et al*., 1989; Kratzschmar *et al*., 1991). Sequence analysis revealed that the vampire bat protease displayed similarities with the human fibrinolytic protease, t-PA. It was soon discovered that this molecule existed as four distinct variants referred to as DSPAα1, α2, β and γ that were encoded by separate genes (Kratzschmar *et al*., 1991; Schleuning and Donner, 2003; Tellgren-Roth *et al*., 2009). Although each variant behaved as a classic plasminogen activator, they were shown to display distinct pharmacokinetic and biochemical properties (Kratzschmar *et al*., 1991; Bringmann *et al*., 1995).

### *Comparison of the DSPA variants with t-PA*

As shown in Figure 1, t-PA harbours five classic domains: a fibronectin (or finger) domain (F), an EGF domain (E) two 'kringle' (K1, K2) domains and a protease domain (P). These domains are important in enabling t-PA to bind to its cofactors and cell surface receptors, engage inhibitors and to recognize and cleave plasminogen (Cesarman-Maus and Hajjar, 2005; Longstaff *et al*., 2011). Another feature worth highlighting is that t-PA exists as an active single-chain species (sct-PA) that can be cleaved by plasmin at a site in the protease domain into a two chain form (tct-PA) (Figure 1). sct-PA and tct-PA have different properties. In the absence of fibrin, sct-PA is less active than tct-PA; however, both forms were shown to activate plasminogen to a similar extent in the presence of fibrin (Loscalzo, 1988). In other words, sct-PA is more fibrin-dependent and fibrin-selective than tct-PA. More recent studies have confirmed that tct-PA has higher enzyme efficiency compared with sct-PA in solution but had similar activities in the presence fibrinogen or cleaved fibrin (Thelwell and Longstaff, 2007). This later study also showed that sct-PA was more susceptible to PAI-1 inhibition in solution, but in the presence of fibrin, PAI-1 inhibition was slower and there was no difference between sct-PA and tct-PA (Thelwell and Longstaff, 2007). While PAI-1 is certainly a major regulator of t-PA in the circulation, an important endogenous inhibitor of t-PA in the CNS is neuroserpin. Neuroserpin, is approximately twofold more efficient at inhibiting tct-PA than sct-PA and is fivefold weaker at blocking u-PA (Hastings *et al*., 1997).

When looking at the structural architecture of the four DSPA molecules, each variant was shown to contain a domain configuration common to t-PA, and the differences between the variants were due nearly exclusively to selective and complete domain deletions. Based on structural and sequencing grounds, DSPAα1 and DSPAα2 are the closest variants to human t-PA as they harbour the same domains as t-PA except for K2, which is absent (Figure 1). These two forms of DSPA have an identical number of amino acids (477, including the leader sequence) with an overall identity of 89%. DSPAβ appears to be the most abundant transcript in




## Figure 1

Schematic representation of t-PA (A) and desmoteplase (DSPAα1) (B) showing the Finger, EGF, Kringle (K) and protease domains. Residues coloured red and noted with an asterisk in t-PA indicate the catalytic triad (His, Asp, Ser). The corresponding residues on DSPAα1 are also indicated with an asterisk and coloured red. Amino acid substitutions in DSPAα1 that differ from t-PA are coloured blue in panel B. The green line adjacent to residues 188–191 (LHST) in panel B indicates the lost plasmin cleavage site. Disulphide bonds are shown as red lines. Panel C insert: Differences between t-PA and DSPAα1 are shown based on the t-PA structure. Substituted residues are shown in red. The catalytic triad (His, Asp, Ser) is again noted with asterisks. CHO: glycosylation site.

bat saliva based on the number of clones detected during the initial screening of the cDNA library (Kratzschmar *et al.*, 1991). This variant harbours only the E, K1 and P domains as it is devoid of the F domain and is 431 amino acids long. DSPAγ is the most truncated variant harbouring only the K1 and P domains and is 394 amino acids long (Table 1). DSPAγ also has the longest 5′-untranslated region and includes a conserved regulatory AP-2/Sp-1 element previously identified in the t-PA gene (Medcalf *et al.*, 1990).

The sole kringle domain common to all four DSPA variants is more related to the K1 domain in t-PA, more than to K2. Consistent with this, the K1 domain of the DSPAs does not contain the lysine binding site, which is a characteristic feature of the K2 domain of t-PA that is used for binding to exposed lysine residues in partially degraded fibrin (van Zonneveld *et al.*, 1986).

In addition to the omission of the K2 domain, each of the DSPA variants lack the plasmin sensitive processing site that is otherwise present in the protease domain of t-PA at position 275 (Figure 1). Hence, none of the DSPA molecules are susceptible to cleavage into a two-chain form. Thus, they are expressed as stable single-chain plasminogen activators (Kratzschmar *et al.*, 1991; Schleuning and Donner, 2003). Hence, these bat-derived plasminogen activators are in fact single-chain proteases with a very low intrinsic activity, but their activity is potentiated enormously by the presence of fibrin. The biochemistry underlying this remarkable feature of DSPA has been documented (Renatus *et al.*, 1997). Analysis of the crystal structure of desmoteplase revealed that, despite being a single-chain protein, its conformation was nonetheless very similar to activated two-chain t-PA (Renatus *et al.*, 1997; Stubbs *et al.*, 1998), yet other structural constraints

## Table 1

Comparison of the structure of t-PA with the plasminogen activators in vampire bats and related bat species

| Species | Plasminogen activator | Structural configuration | Plasmin cleavage site | Fold difference in fibrin selectivity relative to t-PA |
|---|---|---|---|---|
| Human | t-PA | F, E, K1, K2, P | Yes | 1 |
| *D. rotundus* | DSPAα1 | F, E, K1, P | No | 180 |
| *D. rotundus* | DSPAα2 | F, E, K1, P | No | 90 |
| *D. rotundus* | DSPAβ | E, K1, P | No | 3 |
| *D. rotundus* | DSPAγ | K1, P | No | 1.5 |
| *D. youngi* | Single PA variant, related to DSPAα1 | F, E, K1, P[a] | No[b] | Not tested |
| *D. ecaudata* | Single PA variant, related to t-PA | F, E, K1, K2, P[a] | yes | Not tested |
| *C. perspicillata* | Single PA variant, related to t-PA | F, E, K1, K2, P[a] | yes | Not tested |

[a]Based on genomic data (Tellgren-Roth *et al.*, 2009).
[b]Harbours the Ile residue in the plasmin cleavage site, yet the adjacent Arg residue is divergent and therefore unlikely to provide a plasmin cleavage site (see Figure 2).

render the molecule largely inactive in its native state. The switching of this equilibrium status from a zymogen to a fully active form is achieved by fibrin binding. It was proposed that the finger domain-mediated anchorage of the bat protease to fibrin brings together specific surface features of fibrin and the catalytic domain of desmoteplase resulting in the stabilisation of an essential 'salt-bridge'. This in turn shifts the zymogen equilibrium status of desmoteplase towards a fully active conformation (Renatus *et al.*, 1997).

The absence of the plasmin cleavage site has proven to be relevant in the fibrin specificity of the DSPAs (see below). The amino acid sequence identity between the domains of all four DSPA variants with the corresponding domains in t-PA is generally greater than 72% (Kratzschmar *et al.*, 1991). Indeed, the domains within each DSPA variant contain exactly the same number of residues as the corresponding domains in t-PA. This remarkable degree of structural and sequence similarity is a classic example of convergent evolution.

### *Glycosylation sites*

t-PA harbours glycosylation sites at $Asn^{117}$ in K1, $Asn^{184}$ in K2 and $Asn^{448}$ in the P domain that are mostly associated with its clearance from plasma. The glycosylation site in K2 is absent from DSPA due to the complete deletion of this domain. However, the $Asn^{448}$ site is present in all four variants at the same relative position (for DSPAα1, this is at position 362). The $Asn^{117}$ oligomannose site in t-PA is found at the same position in the kringle 1 domain of DSPAα1, while a previously undescribed site with no equivalent in t-PA, is found at position 149 in DSPAα2 and β. The kringle domain of DSPAγ does not harbour any N-glycosylation signal. The functional significance of these variations in glycosylation sites among the DSPA variants is not known, although they are certainly not required for fibrinolytic activity (Schleuning *et al.*, 1992).

### *The distinguishing role of fibrin*

Fibrin is an essential cofactor for t-PA-mediated plasminogen activation. Indeed, the rate at which t-PA can activate plasminogen to plasmin is increased ~550-fold in the presence of fibrin but only eightfold in the presence of fibrinogen (Bringmann *et al.*, 1995). Using the ratio of the rate constants of plasminogen activation in the presence of fibrin (550-fold) and fibrinogen (eightfold), a measure of the relative fibrin-selective increase in t-PA-mediated plasminogen activation can be determined to be 72-fold. This study also assessed the fibrin dependency and fibrin selectivity of plasminogen activation of all four DSPAs (Bringmann *et al.*, 1995). All variants displayed fibrin dependence but differed greatly amongst each other. The ability of DSPAα1 to activate plasminogen was remarkably fibrin-dependent, increasing the catalytic efficiency of plasminogen activation by 102 100-fold. Under the same conditions, the potentiating effect of fibrinogen was only eightfold; hence, the fibrin-selective increase in the activation of plasminogen by DSPAα 1 was 12 900 fold. Similar comparisons with DSPAα2, DSPAβ and DSPAγ yielded fibrin-selective increases in plasminogen activation of 6550, 235 and 90-fold respectively (Bringmann *et al.*, 1995). When comparing the fibrin-selective plasminogen-activating capacity of the four DSPA variants relative to t-PA (i.e. 72-fold), it was revealed that DSPAα1, DSPAα2, DSPAβ and DSPAγ were 180-, 90-, 3- and 1.25-fold more fibrin-selective than t-PA respectively (Table 1).

As mentioned earlier, t-PA exists as a single-chain and as a two-chain active molecule. Cleavage of sct-PA into tct-PA occurs at the plasmin cleavage site in the protease domain of the molecule and alters its fibrin-selectivity. Introduction of a homologous cleavage site into DSPAα1 also reduced its fibrin selectivity by ~12-fold, although it was still more fibrin-selective than t-PA. It was concluded from this and subsequent studies (Renatus *et al.*, 1997) that fibrin dependence and fibrin selectivity of the DSPAs are mediated by fibrin binding, which involves the F domain, undefined determinants within the K and P domains and by the absence of a plasmin-sensitive activation site. Studies on the degree of sensitivity of the DSPA variants to PAI-1 or neuroserpin have not been described.

## Phylogenetic adaptation of *D. rotundus*: the relevance of the K2 domain and the plasmin cleavage site

As mentioned at the outset, there are only three species of bats that have adapted to blood feeding. However, despite their blood-feeding requirement, these three species do not have the same blood preference. The most primitive of these species, *Diphylla ecaudata* feeds exclusively on avian blood (Greenhall *et al*., 1984). *Diaemus youngi*, on the other hand, has a strong preference for avian blood, but it has been reported to also feed on mammalian blood (Greenhall and Schutt, 1996), whereas *D. rotundus* generally feeds on mammalian blood (Cartwright and Hawkey, 1968; Greenhall *et al*., 1983). Differences also exist in the substrate specificities of the fibrinolytic agents in the saliva of these vampire bats. Indeed, the saliva from *D. rotundus* can lyse clots from various mammals, yet it cannot activate avian plasminogen to lyse clots formed from avian blood. Conversely, saliva from *D. youngi* can lyse avian but not mammalian clots (Cartwright and Hawkey, 1968; Cartwright, 1974). It therefore appears that the activation of avian plasminogen is unusual, requiring a unique capability of the plasminogen activator present in *D. youngi*. Hence, in the transition from bird to mammal blood feeding by vampire bats, the plasminogen activator in the saliva of *D. rotundus* had to accommodate this alteration in substrate specificity. In going through this transition, *D. rotundus* evolved four distinct DSPA variants that may also have been a necessary adaptation to allow efficient activation of plasminogen from the different blood sources.

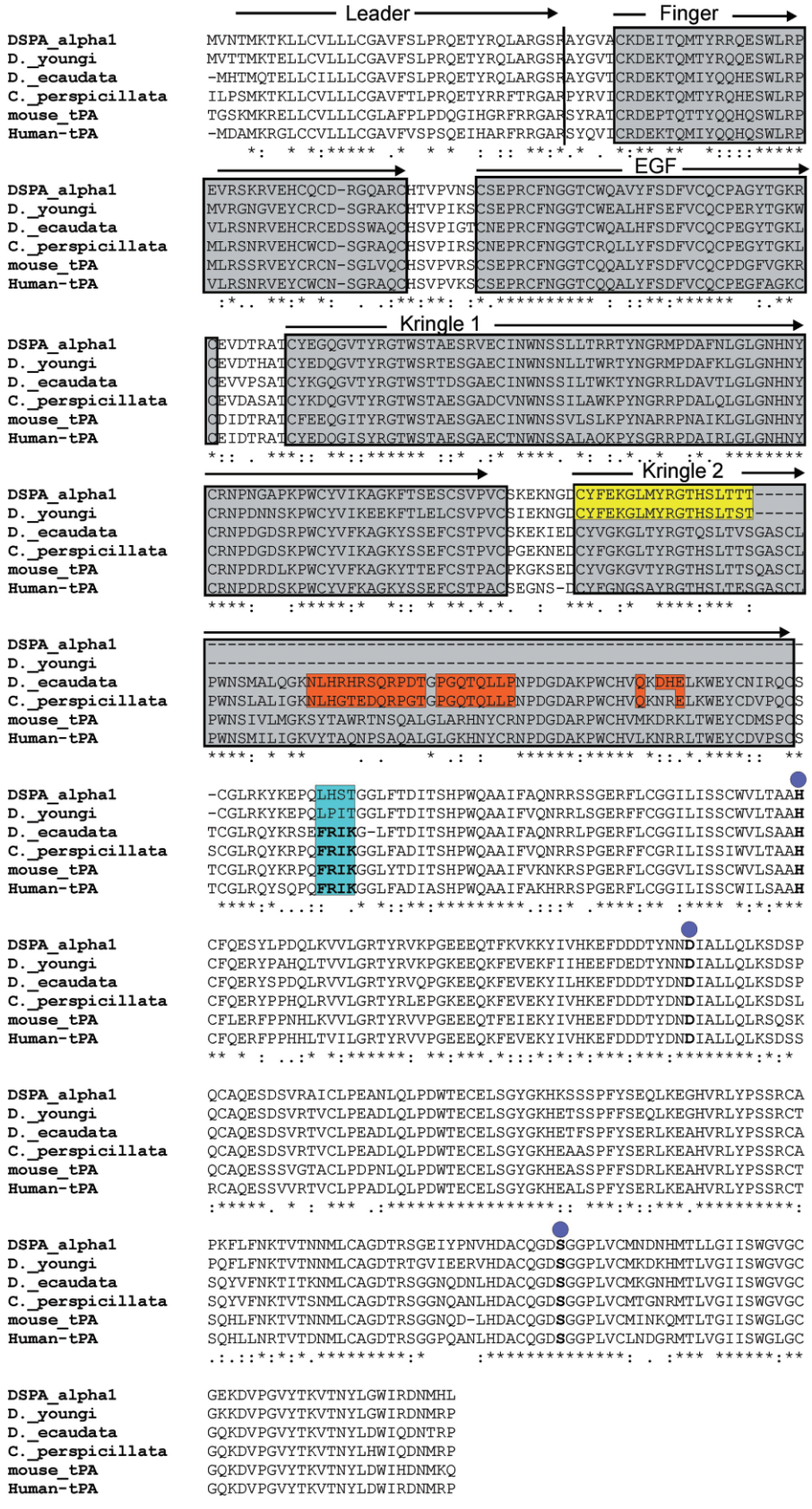
Comparative genomic data of all three species of vampire bats were recently reported (Tellgren-Roth *et al*., 2009). In this study, remarkable differences were reported in the genomic structure of the plasminogen activator genes present in these species that may shed light on their feeding behaviour. Based on these findings, it appears that evolutionary pressure has not only allowed activation of mammalian plasminogen by the plasminogen activators in *D. rotundus* but also fine-tuned them to be optimally effective and selective for fibrin clots formed in mammalian blood.

This genomic analysis confirmed that *D. rotundus* harboured four DSPA variants. No additional variants corresponding to t-PA were found. However, the genomes of the two bird blood-feeding vampire bats, *D. ecaudata* and *D. youngi*, contained only a single plasminogen activator-related gene. Interestingly, the exon composition of this variant in *D. youngi* resembled DSPAα1 in that the exons encoding the K2 domain were absent. The plasmin cleavage site in human t-PA (Phe-Arg-Ile-Lys; single letter code: FRIK) that is notably absent in DSPAα1 (Leu-His-Ser-Thr; LHST) was reported to retained in this species (Leu-Pro-Ile-Thr; LPIT) based on the conservation of the Ile residue (Tellgren-Roth *et al*., 2009), but this is unlikely to provide a plasmin sensitive site due to the loss of the adjacent Arg residue. Hence, this plasmin cleavage site is almost certainly lost in this species as seen in DSPAα1. However, the plasminogen activator species found in the genome of the exclusive avian blood feeder, *D. ecaudata* contained an exon structure akin to t-PA: that is, all t-PA-related exons were present, including exons 8 and 9 encoding the K2 domain, while the plasmin cleavage site (FRIK) was in fact clearly present (Tellgren-Roth *et al*., 2009) (Table 1, Figure 2). Hence, the more primitive blood-feeding vampire bat contains a plasminogen activator most closely related to mammalian t-PA, at least at the genomic level. Whether splice variants exists in this species remains to be determined. The exon structure of one of the insect and fruit-eating bats, *Carollia perspicillata*, was also shown to harbour single plasminogen activator with a genomic structure similar to t-PA, including the plasmin cleavage site. Sequence alignment of the inferred translated product based on the genomic sequence data of *D. ecaudata*, *D. youngi*, *C. perspicillata*, *D. rotundus* as well as mouse and human t-PA is shown in Figure 2. What is evident in this alignment is there is surprising overall domain homology between all of the variants. However, the K2 domains present in *D. ecaudata* and also *C. perspicillata* have stretches of marked sequence divergence (indicated in orange in Figure 2). It is also clear that of these species only *D. rotundus* and *D. youngi* have lost the plasmin cleavage site.

A comparison of the degree of homology between the domains of t-PA with the corresponding domains in plasminogen activators from the various bat species is shown in Table 2. The weaker homology between the K2 domain of the t-PA variant in *D. eucadata* and *C. perspicillata* with the human K2 domain is evident, while the finger domain of DSPAα1 is also less related to the finger domain in human t-PA (57.89%) compared with the other bat PA variants.

The structural basis for the differences in the activation of avian and mammalian plasminogen by the different vampire bat activators is unknown. The absence of the K2 domain in the DSPA variants is not likely to be the reason for their ability to activate mammalian plasminogen, given that t-PA also contains this domain. Nonetheless, the loss of this domain and the absence of the plasmin cleavage site in the evolution of *D. rotundus* have certainly empowered this particular vampire bat with some evolutionary advantage for mammalian blood feeding. Perhaps a clue to this becomes apparent when consideration is given to the differences in the haemostatic system of birds and mammals. The contact system of blood coagulation does not appear to exist in birds, and the avian fibrinolytic system does not contain the plasminogen activator inhibitors, PAI-1 or PAI-2 and also harbours a vastly different u-PA (Sipley *et al*., 1997). The notable absence of PAI-1 in bird blood would permit more efficient fibrinolysis of avian blood clots by the saliva of vampire bats. However, the adaptation of *D. rotundus* to feeding on mammalian blood containing abundant levels of PAI-1 would require a counter-measure. As recently postulated (Tellgren-Roth *et al*., 2009), the loss of the K2 domain in the DSPA variants would be beneficial in this regard, since the loss of the K2 domain in human t-PA has been shown to reduce PAI-1 binding (Thelwell and Longstaff, 2007).

The fact that no additional t-PA-related plasminogen activators exist in the genome of *D. rotundus* implies that one or more of the DSPA variants detected in saliva and/or a u-PA homologue must also reside in the circulatory system of these animals for normal haemostasis. It further remains to be determined what natural inhibitory molecules exist to limit the activity of DSPA *in vivo*. Some inhibitory capacity must exist in the circulation of these vampire bats for normal haemostasis and of course to permit oral wounds to repair,


Leader
Finger
EGF
Kringle 1
Kringle 2
DSPA_alpha1
D._youngi
D._ecaudata
C._perspicillata
mouse_tPA
Human-tPA

## Figure 2

ClustalW analysis was performed to generate the amino acid sequence alignment of human and mouse tissue-type plasminogen activator together with the plasminogen activator variants in *D. rotundus* (DSPAα1 shown), *D. ecaudata*, *D. youngi* and *C. perspicillata*. All sequences were obtained from the NCBI database. The Finger, EGF, Kringle 1 and Kringle 2 domains are boxed, while the protease domain is immediately after the Kringle 2 domain and comprises most of the molecule. Regions coloured in orange in the *D. ecaudata* and *C. perspicillata* sequences within the Kringle 2 domain indicate marked deviation from mammalian t-PA. The region within the second kringle (yellow box) is an untranslated sequence in DSPAα1, which is also seen in *D. youngi*. The plasmin cleavage site FRIK (or lack thereof) is shown in the blue box. The three dark blue dots above the histidine (H), aspartic acid (D) and serine (S) residues indicate the conserved catalytic triad. Asterisks and dots below the sequence indicate degree of sequence conservation between all variants as derived from ClustalW analysis.

## Table 2

Comparison of the amino acid homology of the structural domains of the various bat-derived plasminogen activators with human t-PA

| | *D. rotundus* (DSPAα1), % | *D. youngi*, % | *D. eucadata*, % | *C. perspicillata*, % |
|---|---|---|---|---|
| t-PA Finger domain (38 residues) | 57.89 | 70.4 | 81.57 | 84.21 |
| t-PA EGF domain (34 residues) | 82.35 | 76.47 | 82.35 | 82.35 |
| t-PA K1 domain (82 residues) | 69.51 | 68.29 | 78.05 | 84.14 |
| t-PA K2 domain (82 residues) | – | – | 56.09 | 63.41 |
| t-PA Protease domain (264 residues) | 77.27 | 75.00 | 83.33 | 85.61 |
| *Overall domain identity with t-PA* | 74.40 | 73.20 | 77.80 | 81.40 |

which would otherwise be difficult in the presence of saliva containing such an abundant fibrinolytic arsenal.

While further research is needed to unravel these questions concerning the endogenous fibrinolytic system in the vampire bat, the fact that the vampire bat-derived plasminogen activators had proved to harbour such remarkable fibrin selectivity prompted their consideration for development as thrombolytic drugs.

## t-PA versus desmoteplase in animal models of arterial thrombosis

The more fibrin-selective the protease, the higher the likelihood that it will activate clot-bound rather than circulating plasminogen. Since DSPAα1 (desmoteplase) was shown to be the most fibrin-selective of all four DSPA variants and ~180-fold more fibrin-selective than t-PA, it was ultimately selected for clinical development. It should also be noted that DSPAα2 ('Bat-PA') was cloned by another group (Gardell *et al.*, 1989) and later considered for clinical development. Although extensive preclinical studies were undertaken with bat-PA (Gardell *et al.*, 1990; 1991; Bergum and Gardell, 1992; Hare and Gardell, 1992; Gardell and Friedman, 1993; Mellott *et al.*, 1995; Montoney *et al.*, 1995), this clinical programme was subsequently abandoned.

Desmoteplase was initially placed under clinical development for the treatment of patients with myocardial infarction in the early 1990s. Numerous studies were published during this period that compared the relative potency, efficacy and safety of desmoteplase with t-PA and other plasminogen activators (Gulba *et al.*, 1995; Sakharov *et al.*, 1999). In brief, the fibrin-selective nature of desmoteplase was shown to induce effective lysis of human plasma clots over a large concentration range (3500-fold), which was far greater than for t-PA (35-fold) and other fibrin-dependent plasminogen activators (e.g. TNK-tPA and staphylokinase) (Sakharov *et al.*, 1999).

Rabbit (Muschick *et al.*, 1993), rodent (Witt *et al.*, 1992) and canine (Witt *et al.*, 1994) models of thrombosis were used to compare the efficacy of desmoteplase with recombinant t-PA. In a model of myocardial infarction in dogs, desmoteplase, when delivered at 25, 50 or 100 $\mu g \cdot kg^{-1}$ resulted in 100% incidence of patency after 37, 23 and 18 min, respectively, while t-PA administered at 63 and 125 $\mu g \cdot kg^{-1}$ recanalized arteries in 33% and 50% of cases within 40 min (Witt *et al.*, 1994). In a rat model of pulmonary embolism, desmoteplase-induced thrombolysis was achieved faster than with t-PA (Witt *et al.*, 1992). Moreover, t-PA but not desmoteplase was shown to cause a reduction in levels of fibrinogen and plasminogen. The lack of systemic consumption of coagulation proteins by desmoteplase further underscored the potential therapeutic benefit of a highly fibrin-selective thrombolytic agent.

Another relevant parameter is that desmoteplase has a substantially longer plasma half-life compared with t-PA. Pharmacokinetic analysis of desmoteplase in rats using an ELISA approach (Hildebrand *et al.*, 1995) showed lower total clearance and a longer terminal half-life in comparison with t-PA (Hildebrand *et al.*, 1996). Furthermore, bolus administration of desmoteplase (0.01–0.05 mg) to healthy volunteers resulted in a biphasic clearance curve ($t_{1/2\alpha}$ of 4 min, $t_{1/2\beta}$ of 2.3–2.7 h), the second phase being responsible for the elimination of 83% of the molecule (Gulba *et al.*, 1997) (2.8 h vs. 2.5–4 min for t-PA), which enables its administration to patients as a bolus i.v. injection (Van de Werf, 1999).

Despite these data, the intended development of desmoteplase for the treatment of patients with myocardial infarction was discontinued. However, these early preclinical studies laid the foundation for its subsequent re-emergence as a treatment of patients with acute ischaemic stroke.

## Thrombolysis in ischaemic stroke: limitations and challenges

t-PA is currently the only thrombolytic agent approved for use in patients with acute ischaemic stroke. However, the clinical benefit of t-PA has limitations: t-PA is effective if given to ischaemic stroke patients within 3 h of stroke onset (Hacke *et al.*, 2004), although this has now been extended to 4.5 h (Hacke *et al.*, 2008; Davis and Donnan, 2009). Delivery after this time period is associated with an increased risk of symptomatic intracerebral haemorrhage (ICH) (Lees *et al.*, 2010). This restricted time window means that only a small percentage of ischaemic stroke patients actually receive t-PA. Indeed, the vast majority of stroke patients receive no thrombolytic therapy at all, since they fall outside this critical time window. The improved feature of desmoteplase over t-PA with respect to fibrin selectivity, lack of systemic consumption of plasminogen or fibrinogen, coupled with its longer half-life (allowing bolus delivery), raised the possibility that it could be used effectively in patients with ischaemic stroke and, more importantly, over an extended time frame.

Fibrin selectivity is still viewed as the Holy Grail for a plasminogen activator in the context of thrombolysis; however, this feature alone may not necessarily be sufficient for improved outcomes in patients with ischaemic stroke. While fibrin selectivity of a thrombolytic agent is certainly advantageous, another parameter that needs to be considered is its potential to modulate brain function, particularly its ability to alter permeability of the blood–brain barrier (BBB) and to promote neurotoxicity, areas that were completely unforeseen and unimaginable in the mid-1990s during the growth phase of thrombolytic development.

### *Neurotoxicity, the BBB and thrombolysis*

It is now well established that endogenous t-PA is not only a fibrinolytic protease but is also implicated in many aspects of the CNS via plasmin-dependent and plasmin-independent pathways (Yepes and Lawrence, 2004; Samson and Medcalf, 2006) and in some cases even without the need for proteolysis at all (Rogove *et al.*, 1999). Its role in memory development (Baranes *et al.*, 1998), synaptic plasticity (Seeds *et al.*, 1995), stress and anxiety (Pawlak *et al.*, 2003; 2005b) has now generated much interest in other fields of research.

Perhaps the most startling revelation was that the observation that t-PA$^{-/-}$ mice were relatively resistant excitotoxic injury (Tsirka *et al.*, 1995). In this study, t-PA$^{-/-}$ mice were shown to be resistant to kainic acid-induced injury of hippocampal neurons, while reintroduction of t-PA restored this form of neurotoxicity (Tsirka *et al.*, 1996). t-PA-induced neuronal injury was a plasmin-dependent process, since plasminogen$^{-/-}$ mice were also resistant to kainic acid-induced hippocampal injury. t-PA$^{-/-}$ mice were also shown to harbour resistance to other injury paradigms including cerebral ischaemia (Wang *et al.*, 1998) and traumatic brain injury (Mori *et al.*, 2001), thereby implicating endogenous t-PA in the cascade of events leading to neurotoxicity. These revelations initiated intense research to understand the means by which t-PA was impacting on brain function. While this has not come without controversy (Matys and Strickland, 2003), the general consensus of opinion is that t-PA potentiates neurotoxicity by promoting NMDA receptor (NMDAR) function by engaging one or more of the NMDAR subunits, depending on the injury model (Nicole *et al.*, 2001; Kilic *et al.*, 2005; Pawlak *et al.*, 2005a; Norris and Strickland, 2007; Baron *et al.*, 2010) or acting in concert with other co-receptors including low-density lipoprotein receptors (LDLR; LRP-1) (Samson *et al.*, 2008) and protease activated receptors (PAR-1) (Mannaioni *et al.*, 2008; Samson *et al.*, 2008) and the PDGFR-α receptor (Su *et al.*, 2008) (below). A key question is the role of plasmin in this neurotoxic process: while plasminogen$^{-/-}$ mice have been shown to be as resistant to excitotoxic injury as t-PA$^{-/-}$ mice, other studies from one group have provided evidence that t-PA can directly cleave and activate the NR1 subunit of the NMDAR (Nicole *et al.*, 2001). However, this is a controversial issue with others questioning this mode of action (Matys and Strickland, 2003; Samson *et al.*, 2008).

While the implication of these studies has instilled concerns over the clinical use of t-PA, it is important to bear in mind that t-PA does not promote neurotoxicity at all to the uninjured brain. I.v. delivery of t-PA in uninjured mice has been shown to traverse the BBB without any detectable consequences (Benchenane *et al.*, 2005). Intraventricular injection of t-PA was also shown to increase BBB permeability (Su *et al.*, 2008), but whether this caused impairment was not addressed. Furthermore, direct injection of t-PA into the striatal region of the mouse brain did not create any detectable lesion (Liberatore *et al.*, 2003). That t-PA *per se* is safe under normal conditions is also highlighted in studies using transgenic mice that selectively overexpress t-PA in neurons. These mice (referred to as 'T4 mice') were engineered to produce greater than 10-fold higher levels of t-PA in the brain. They displayed no obvious CNS deficiencies (Madani *et al.*, 1999) but were distinguished by enhanced learning capabilities and improved long-term potentiation (LTP) (Madani *et al.*, 1999). This positive effect of t-PA with learning is consistent with other studies that have linked t-PA deficiency with impaired LTP and memory (Qian *et al.*, 1993; Baranes *et al.*, 1998). One would anticipate, however, that the increased availability of endogenous t-PA in these 'smart' T4 mice would make them more sensitive to ischaemic or excitotoxic injury. On the other hand, the levels of t-PA present in T4 mice, although 10–17-fold higher than in wild-type littermates (Sashindranath *et al.*, 2011), is still less than the levels of exogenous t-PA delivered in various injury paradigms, making predictions of toxicity or otherwise in these mice difficult. In any case, the discrepancy between the effect of t-PA in normal and in injured brain scenarios implies that t-PA toxicity is injury-dependent.

### *Neurotoxicity: t-PA versus desmoteplase*

Despite controversy in the field, it appears that plasmin formation plays a causal role in the effector arm of t-PA-dependent neurotoxicity. It would therefore be anticipated

that *any* plasminogen activator capable of activating plasminogen within the lesioned brain would confer the same degree of neuronal injury as t-PA. However, this is not the case. Liberatore *et al*. (2003) revealed that desmoteplase could not substitute for t-PA to promote neuronal injury since hippocampal neurons of the t-PA$^{-/-}$ mice were still resistant to kainic acid-induced injury when desmoteplase was administered into the lesion even at a 10-fold molar excess over t-PA. These authors also showed that the co-injection of t-PA directly into the striatal region (Liberatore *et al*., 2003) or i.v. (Reddrop *et al*., 2005) potentiated NMDA-induced lesion volume, whereas desmoteplase had no effect in either case despite the fact that desmoteplase was shown to have entered the parenchyma. A similar result was seen in an independent study where i.v. administration of t-PA, but not desmoteplase, promoted excitotoxic injury following intrastriatal injection of NMDA (Lopez-Atalaya *et al*., 2007).

t-PA was also shown to potentiate NMDA-induced neuronal cell death *in vitro*, whereas desmoteplase had no damaging consequences (Reddrop *et al*., 2005). Surprisingly, desmoteplase was shown to block the neurotoxic effect of t-PA, suggesting that the potentiation of excitoxicity by t-PA also required receptor engagement (presumably an NMDAR subunit) in addition to proteolysis, and that desmoteplase competed with this action of t-PA. Based on these collective findings, it was concluded that desmoteplase does not harbour the neurotoxic capabilities of t-PA.

Why is it that desmoteplase lacks neurotoxicity, since plasmin formation has previously been shown to be a necessary event for t-PA-induced injury? Since desmoteplase is remarkably fibrin-dependent, it was postulated that the non-neurotoxic action of desmoteplase was a consequence of a lack of its requisite cofactor, fibrin, within the lesioned area (Liberatore *et al*., 2003). Another possibility is that t-PA toxicity requires not only plasmin formation but also the engagement of other cell surface receptors or proteins. t-PA is known to associate with other cells of the CNS via specific cell surface receptors including annexin II/p11 (Cesarman-Maus and Hajjar, 2005; Kwon *et al*., 2005) and LDLR-related receptor (LRP) (Orth *et al*., 1994). As mentioned earlier, t-PA can also activate PDGF-CC and can bind to various subunits of the NMDAR. t-PA-mediated plasminogen activation is not only potentiated by fibrin but also by prion proteins (Ellis *et al*., 2002; Epple *et al*., 2004) amyloid-β peptides (Kingston *et al*., 1995) and misfolded proteins that occur in cells following injury (Samson *et al*., 2009). Regarding these t-PA binding proteins, desmoteplase-mediated plasminogen activation is only poorly influenced by prion protein (Epple *et al*., 2004) and amyloid-β proteins (Kruithof and Schleuning, 2004).

Compared with t-PA, desmoteplase lacks the K2 domain and the plasmin cleavage site in the protease domain. The binding of t-PA to the NR1 subunit, prion protein and PDGF-CC have been shown to require the K2 domain that contains the lysine-binding site (Epple *et al*., 2004; Fredriksson *et al*., 2005; Lopez-Atalaya *et al*., 2008), although its interaction with amyloid requires the finger domain (Maas *et al*., 2008). Hence, the lack of the K2 domain in desmoteplase most likely explains its inability to engage most of these proteins and by inference explains its lack of neurotoxicity. While there is merit in this assertion, there is one inconsistency. If t-PA neurotoxicity was solely dependent of the K2 domain, then it would be expected that K2-dependent neurotoxicity would not be inhibited by proteins that do not possess the K2 domain. However, despite lacking the K2 domain, desmoteplase inhibited t-PA-mediated neurotoxicity *in vitro* (Reddrop *et al*., 2005). Hence, while the K2 domain features as the causative domain in some aspects of t-PA activity in the CNS, other regions in the t-PA molecule may also play contributing roles in this process.

## t-PA and the BBB

Given the potential of t-PA to promote neurotoxicity, it may seem intuitive that this would be reflected with loss of structural integrity of the neurovascular unit. While this might certainly be expected, recent studies have also indicated that this effect of t-PA can in fact be an active process and not merely a consequence of cell death. t-PA has been shown to promote dilation of the cerebral vasculature (Armstead *et al*., 2005) and can cross the BBB via an LDLR mechanism of transcytosis without altering BBB integrity (Benchenane *et al*., 2005). One study however, demonstrated that t-PA could increase permeability of the neurovascular unit once it had gained access to the parenchyma (Su *et al*., 2008). A more recent study confirmed the ability of t-PA to increase cerebrovascular permeability independent of its catalytic activity (Abu Fanne *et al*., 2010). Intraventricular injection of t-PA into the mouse brain was shown to cause an increase in cerebrovascular permeability within 1 h in both wild-type and plasminogen$^{-/-}$ mice (Su *et al*., 2008), implicating a plasmin-independent process. Furthermore, i.v. delivery of t-PA into normal mice had no effect on permeability (which contrasted with the rat data described above) (Abu Fanne *et al*., 2010); however, if mice were first subjected to an ischaemic stroke, then delivered t-PA i.v., an increase in extravasation was seen (Su *et al*., 2008). On this basis, it seemed therefore that t-PA was capable of promoting neurotoxicity once it had gained access to the brain parenchyma. While this effect of t-PA was unrelated to its ability to activate plasminogen, it was shown by this group that the t-PA-mediated increase in permeability via its direct proteolytic cleavage and activation of the latent form of PDGF-CC (Fredriksson *et al*., 2004), allowing this growth factor to engage its receptor (PDGFR-α receptor) and initiate a signalling pathway essential for this effect. Indeed, addition of antibodies against the PDGFR-α receptor blocked the ability of t-PA to promote extravasation (Su *et al*., 2008; 2009).

Do these findings have any bearing of the use of t-PA in patients with ischaemic stroke? It is tempting to draw a direct link between the increased risk of ICH seen in patients administered t-PA and the potential of t-PA to enhance permeability and/or to promote neurotoxicity. In this context, it is relevant to point out two independent clinical studies have reported that t-PA can indeed alter cerebrovascular permeability in stroke patients (Kidwell *et al*., 2008; Kassner *et al*., 2009).

### *Desmoteplase versus t-PA at the BBB*

Up until now, all animal models that have been used to compare desmoteplase with t-PA in brain injury paradigms

have provided no evidence for a deleterious effect of desmoteplase. This is almost certainly a consequence of the inability of desmoteplase to engage neurotoxic pathways. Its potential modulatory effects on BBB permeability, independent of neurotoxicity, have also been evaluated in one study (Lopez-Atalaya *et al*., 2007). In this study, both t-PA and desmoteplase were shown to pass directly through endothelial cells via transcytosis *in vitro* but without any effect on permeability. Moreover, desmoteplase was also shown to inhibit this ability of t-PA to transverse bovine endothelial cells under normoxic conditions (Lopez-Atalaya *et al*., 2007). I.v. administration of t-PA was also shown to potentiate the degree of excitotoxic injury inflicted by intrastriatal injection of the glutamate analogue, NMDA, thereby confirming previous reports (Reddrop *et al*., 2005) showing that i.v. delivered t-PA can access the parenchyma with damaging consequences. Delivery of desmoteplase via the i.v. route did not potentiate excitotoxic injury but was capable of blocking the damaging effect of i.v. delivered t-PA. Although this study implicated the importance of endothelial LDLRs for the transcytosis of both t-PA and desmoteplase under normoxic conditions, under hypoxic conditions, the transcytotic transfer of t-PA was independent of LDLRs, thereby implicating a distinct process under hypoxic conditions. Although this study implicated LRP-1 as the pertinent LDLR, this was not directly proven since selective LRP-1 inhibitors were not used to verify this claim. Other LDLRs may indeed be worth considering given that LRP-1, while expressed on neurons, is only poorly expressed, if at all, on endothelial cells within the human neurovascular unit (Wolf *et al*., 1992; Tooyama *et al*., 1995). Another inconsistency in the field relates to whether t-PA can indeed increase permeability of the BBB. Although the report by Lopez-Atalaya *et al*. indicated that i.v. delivered t-PA (and desmoteplase) can enter the parenchyma without any deleterious consequences, other studies have shown that direct intraventricular delivery of t-PA provokes extravasation (Su *et al*., 2008; 2009) via activation of PDGF-CC. Desmoteplase was not assessed in this study, and its effect or otherwise on the activation of PDGF-CC is also not known. A schematic representation of the effects of t-PA and desmoteplase on the neurovascular unit is shown in Figure 3.

## The clinical experience

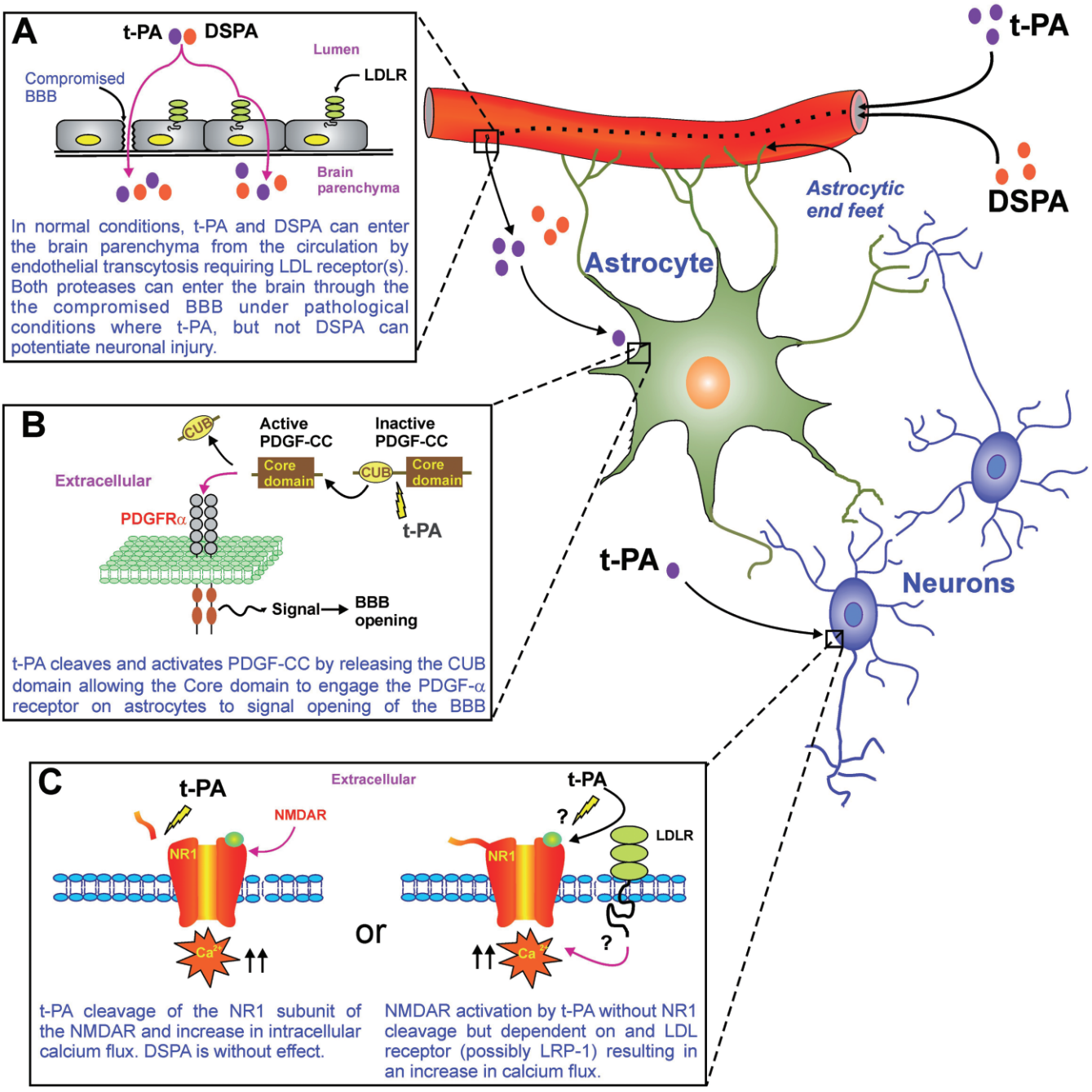
A clinical programme was initiated in 1999, and since this time, three separate clinical trials (two phase II and one phase III) have been conducted to assess the effectiveness of desmoteplase in patients with ischaemic stroke over a comparatively later time frame (i.e. 3–9 h) post-stroke onset (Hacke *et al*., 2005; 2009; Furlan *et al*., 2006). Results of the two phase II studies were particularly promising with improvement in reperfusion and in clinical outcome after 90 days. The results from these two trials prompted a phase III study (Desmoteplase in Acute ischaemic Stroke, 'DIAS-2'). While this trial demonstrated the safety of desmoteplase at later time points post stroke, DIAS-2 failed to demonstrate efficacy in patients with ischaemic stroke. This surprising negative outcome generated a number of discussions and raised many questions (Donnan and Davis, 2007; Liebeskind, 2009). One explanation for the lack of efficacy in DIAS-2 may have related to baseline strokes being less severe in the DIAS-2 trial, resulting in an unusually high placebo response (46%) at the 90 day follow-up time point (Soehngen *et al*., 2010). The placebo response was lower in the previous two phase II trials: DIAS-1, 22% (Hacke *et al*., 2005) and DEDAS, 25% (Furlan *et al*., 2006). Moreover, *post hoc* analyses of the DIAS-2 data showed that when patients had a proximal cerebral vessel occlusion or high-grade stenosis on baseline angiography, a positive response for desmoteplase was shown (Hacke *et al*., 2009). Based on these trends, new clinical trials commenced in 2009 (DIAS III and DIAS IV), and results of these trial are eagerly awaited.

## Conclusion

The saliva of the common vampire bat, *D. rotundus*, was shown to harbour a number of factors that influence haemostasis and fibrinolysis. The only saliva-based protein that has been fully characterized and explored for any therapeutic potential has been desmoteplase. Although the saliva of *D. rotundus* has been shown to possess other modifiers of the haemostatic system (Hawkey, 1967; Cartwright, 1974; Apitz-Castro *et al*., 1995), there is little doubt that other unique substances remain to be discovered in the vampire bat that might offer new opportunities for basic research and potential therapeutic development.

The arsenal of fibrinolytic proteins in *D. rotundus* has been of particular interest to this field. The recent comparative studies on the plasminogen activators in the other two blood-feeding bats (*D. youngi* and *D. ecaudata*) have been most revealing and have lead to the conclusion that DSPAα1, α2, β and γ that lack the K2 domain and the plasmin cleavage site have been tailored by evolution for the purpose of removing mammalian blood clots as efficiently as possible with minimal interference from exogenous fibrinolytic inhibitors. Perhaps serendipitously, the features that have empowered DSPAα1 with targeted and efficient fibrinolysis have also had the desirable consequence of minimal off-target effects, particularly in the brain. Given the similarity of the single variant in *D. youngi* to DSPAα1 (both variants are devoid of the K2 domain and share an overall domain amino acid identity of 83.01%), this variant is likely to harbour similar properties as DSPAα1. The properties of the fibrinolytic moiety in the exclusive bird blood feeder, *D. ecaudata*, are presently unknown.

The remarkable fibrin-selective feature of DSPAα1 (desmoteplase) provided the initial trigger for its clinical development for thrombolysis before its effects or otherwise in the brain were known. While t-PA is still the most widely used thrombolytic agent for patients with myocardial infarction and ischaemic stroke, its use in patients with ischaemic stroke is limited due to its ability to increase the risk of intracerebral haemorrhage. More recent findings have established an unforeseen link between t-PA and the potentiation of neuronal injury, a feature that is now known to be absent in desmoteplase. These differences have provided much anticipation for its use in patients with ischaemic stroke. Although seemingly safe on many grounds, definitive evidence for its efficacy in patients with ischaemic stroke is yet to be formally provided. Aside from its clinical potential, the lack of




## Figure 3

Comparison between t-PA and desmoteplase on the neurovascular unit. Insert A: Under normoxic conditions, t-PA or desmoteplase (DSPA) delivered i.v. can enter the brain parenchyma via LDLR -dependent endothelial cell transcytosis (see text for further details). During pathological conditions, both proteases can enter the brain due to the compromised BBB. Insert B: Once in the parenchyma and during pathological conditions, t-PA, but not DSPA, can promote opening of the BBB. For t-PA, this occurs via activation of PDGF-CC and signalling through the PDGF-α receptor. The action of DSPA on PDGF-CC is not known. Insert C: t-PA can also potentiate neuronal injury by acting on NMDARs. The mechanistic basis for this damaging effect of t-PA during neuronal injury is an area of controversy, whereby t-PA has been proposed to (i) directly cleave the NR1 subunit of the NMDAR promoting calcium influx, or (ii) promote calcium flux in an LDLR -dependent manner (which is generally presumed to be LRP-1); t-PA also needs to be proteolytically active, but NR1 cleavage is not involved. DSPA has no effect on the NMDAR and cannot potentiate calcium flux. Endogenous t-PA released from neurons can also modulate brain function and promote neurotoxicity (not shown in figure).

neurotoxicity of desmoteplase as derived from *in vitro* and *in vivo* studies has provided novel insights in to the means by which mammalian t-PA modulates brain function, which has spawned a new area in the field of plasminogen activator research. Whether these data can be used to engineer t-PA to behave more like desmoteplase is also an approach that might be worth considering.

## Acknowledgements

The author would like to thank past and present members of the Fibrinolysis and Gene Regulation Laboratory at the Australian Centre for Blood Diseases (Monash University) for their contributions to this topic over the past decade. The author also acknowledges the help of Dr Sarah Boyd for advice concerning sequence comparisons of the plasminogen activator variants.

## Conflict of interest

The author states that there is no conflict of interest.

## References

Abu Fanne R, Nassar T, Yarovoi S, Rayan A, Lamensdorf I, Karakoveski M *et al*. (2010). Blood-brain barrier permeability and tPA-mediated neurotoxicity. Neuropharmacology 58: 972–980.

Apitz-Castro R, Beguin S, Tablante A, Bartoli F, Holt JC, Hemker HC (1995). Purification and partial characterization of draculin, the anticoagulant factor present in the saliva of vampire bats (Desmodus rotundus). Thromb Haemost 73: 94–100.

Armstead WM, Cines DB, Higazi AA (2005). Plasminogen activators contribute to impairment of hypercapnic and hypotensive cerebrovasodilation after cerebral hypoxia/ischemia in the newborn pig. Stroke 36: 2265–2269.

Baranes D, Lederfein D, Huang YY, Chen M, Bailey CH, Kandel ER (1998). Tissue plasminogen activator contributes to the late phase of LTP and to synaptic growth in the hippocampal mossy fiber pathway. Neuron 21: 813–825.

Baron A, Montagne A, Casse F, Launay S, Maubert E, Ali C *et al*. (2010). NR2D-containing NMDA receptors mediate tissue plasminogen activator-promoted neuronal excitotoxicity. Cell Death Differ 17: 860–871.

Benchenane K, Berezowski V, Ali C, Fernandez-Monreal M, Lopez-Atalaya JP, Brillault J *et al*. (2005). Tissue-type plasminogen activator crosses the intact blood-brain barrier by low-density lipoprotein receptor-related protein-mediated transcytosis. Circulation 111: 2241–2249.

Bergum PW, Gardell SJ (1992). Vampire bat salivary plasminogen activator exhibits a strict and fastidious requirement for polymeric fibrin as its cofactor, unlike human tissue-type plasminogen activator. A kinetic analysis. J Biol Chem 267: 17726–17731.

Bier OE (1932). Action anticoagulante et fibrinolytique de l'extract des glands salivaires d'une chauve-souris hematophage (*Desmodus rufus*). C R Soc Biol (Paris) 110: 129–131.

Bringmann P, Gruber D, Liese A, Toschi L, Kratzschmar J, Schleuning WD *et al*. (1995). Structural features mediating fibrin selectivity of vampire bat plasminogen activators. J Biol Chem 270: 25596–25603.

Cartwright T (1974). The plasminogen activator of vampire bat saliva. Blood 43: 317–326.

Cartwright T, Hawkey C (1968). Activation of the blood fibrinolytic mechanism in birds by saliva of the vampire bat (*Diaemus youngi*). J Physiol 201: 45P–46P.

Cesarman-Maus G, Hajjar KA (2005). Molecular mechanisms of fibrinolysis. Br J Haematol 129: 307–321.

Davis SM, Donnan GA (2009). 4.5 hours: the new time window for tissue plasminogen activator in stroke. Stroke 40: 2266–2267.

Donnan GA, Davis SM (2007). Life after DIAS II. Int J Stroke 2: 236–237.

Ellis V, Daniels M, Misra R, Brown DR (2002). Plasminogen activation is stimulated by prion protein and regulated in a copper-dependent manner. Biochemistry 41: 6891–6896.

Epple G, Schleuning WD, Kettelgerdes G, Kottgen E, Gessner R, Praus M (2004). Prion protein stimulates tissue-type plasminogen activator-mediated plasmin generation via a lysine-binding site on kringle 2. J Thromb Haemost 2: 962–968.

Fernandez AZ, Tablante A, Beguin S, Hemker HC, Apitz-Castro R (1999). Draculin, the anticoagulant factor in vampire bat saliva, is a tight-binding, noncompetitive inhibitor of activated factor X. Biochim Biophys Acta 1434: 135–142.

Fredriksson L, Li H, Fieber C, Li X, Eriksson U (2004). Tissue plasminogen activator is a potent activator of PDGF-CC. EMBO J 23: 3793–3802.

Fredriksson L, Ehnman M, Fieber C, Eriksson U (2005). Structural requirements for activation of latent platelet-derived growth factor CC by tissue plasminogen activator. J Biol Chem 280: 26856–26862.

Furlan AJ, Eyding D, Albers GW, Al-Rawi Y, Lees KR, Rowley HA *et al*. (2006). Dose Escalation of Desmoteplase for Acute Ischemic Stroke (DEDAS): evidence of safety and efficacy 3 to 9 hours after stroke onset. Stroke 37: 1227–1231.

Gardell SJ, Friedman PA (1993). Vampire bat salivary plasminogen activator. Methods Enzymol 223: 233–249.

Gardell SJ, Duong LT, Diehl RE, York JD, Hare TR, Register RB *et al*. (1989). Isolation, characterization, and cDNA cloning of a vampire bat salivary plasminogen activator. J Biol Chem 264: 17947–17952.

Gardell SJ, Hare TR, Bergum PW, Cuca GC, O'Neill-Palladino L, Zavodny SM (1990). Vampire bat salivary plasminogen activator is quiescent in human plasma in the absence of fibrin unlike human tissue plasminogen activator. Blood 76: 2560–2564.

Gardell SJ, Ramjit DR, Stabilito II, Fujita T, Lynch JJ, Cuca GC *et al*. (1991). Effective thrombolysis without marked plasminemia after bolus intravenous administration of vampire bat salivary plasminogen activator in rabbits. Circulation 84: 244–253.

Greenhall AM, Schutt WA (1996). Diaemus youngi. Mamm Species 533: 1–7.

Greenhall AM, Joermann G, Schmidt U, Seidel MR (1983). Desmodus rotundus. Mamm Species 202: 1–6.

Greenhall AM, Schmidt U, Joermann G (1984). Diphylla eucadata. Mamm Species 227: 1–3.

Gulba DC, Praus M, Witt W (1995). DSPA alpha – Properties of the plasminogen activators on the Vampire bat *Desmodus rotundus*. Fibrinolysis 9: 91–96.

Gulba DC, Praus M, Dechend R, Hauck S, Mahler M, Zitvz R *et al*. (1997). Update on the toxicology and pharmacology of rDSPA alpha 1 (Bat-PA) in animals and humans. Fibrinol Proteol 11: 55–62.

Hacke W, Donnan G, Fieschi C, Kaste M, von Kummer R, Broderick JP *et al*. (2004). Association of outcome with early stroke treatment: pooled analysis of ATLANTIS, ECASS, and NINDS rt-PA stroke trials. Lancet 363: 768–774.

Hacke W, Albers G, Al-Rawi Y, Bogousslavsky J, Davalos A, Eliasziw M *et al*. (2005). The Desmoteplase in Acute Ischemic Stroke Trial (DIAS): a phase II MRI-based 9-hour window acute stroke thrombolysis trial with intravenous desmoteplase. Stroke 36: 66–73.

Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D *et al*. (2008). Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. N Engl J Med 359: 1317–1329.

Hacke W, Furlan AJ, Al-Rawi Y, Davalos A, Fiebach JB, Gruber F *et al*. (2009). Intravenous desmoteplase in patients with acute ischaemic stroke selected by MRI perfusion-diffusion weighted imaging or perfusion CT (DIAS-2): a prospective, randomised, double-blind, placebo-controlled study. Lancet Neurol 8: 141–150.

Hare TR, Gardell SJ (1992). Vampire bat salivary plasminogen activator promotes robust lysis of plasma clots in a plasma milieu without causing fluid phase plasminogen activation. Thromb Haemost 68: 165–169.

Hastings GA, Coleman TA, Haudenschild CC, Stefansson S, Smith EP, Barthlow R *et al*. (1997). Neuroserpin, a brain-associated inhibitor of tissue plasminogen activator is localized primarily in neurons. Implications for the regulation of motor learning and neuronal survival. J Biol Chem 272: 33062–33067.

Hawkey C (1966). Plasminogen activator in saliva of the vampire bat Desmodus rotundus. Nature 211: 434–435.

Hawkey C (1967). Inhibitor of platelet aggregation present in saliva of the vampire bat Desmodus rotundus. Br J Haematol 13: 1014–1020.

Hildebrand M, Bunte T, Bringmann P, Schutt A (1995). Development of an ELISA for the measurement of DSPA$\alpha_1$ (Desmodus rotundus salivary plasminogen activator) in plasma and its application to investigate pharmacokinetics in monkeys. Fibrinolysis 9: 107–111.

Hildebrand M, Bhargava AS, Bringmann P, Schutt A, Verhallen P (1996). Pharmacokinetics of the novel plasminogen activator *Desmotus rotundus* plasminogen activator and extrapolation to man. Fibrinolysis 10: 269–276.

Kassner A, Roberts TP, Moran B, Silver FL, Mikulis DJ (2009). Recombinant tissue plasminogen activator increases blood-brain barrier disruption in acute ischemic stroke: an MR imaging permeability study. AJNR Am J Neuroradiol 30: 1864–1869.

Kidwell CS, Latour L, Saver JL, Alger JR, Starkman S, Duckwiler G *et al*. (2008). Thrombolytic toxicity: blood brain barrier disruption in human ischemic stroke. Cerebrovasc Dis 25: 338–343.

Kilic E, Kilic U, Bahr M, Hermann DM (2005). Tissue plasminogen activator-induced ischemic injury is reversed by NMDA antagonist MK-801 in vivo. Neurodegener Dis 2: 49–55.

Kingston IB, Castro MJ, Anderson S (1995). In vitro stimulation of tissue-type plasminogen activator by Alzheimer amyloid beta-peptide analogues. Nat Med 1: 138–142.

Kratzschmar J, Haendler B, Langer G, Boidol W, Bringmann P, Alagon A *et al*. (1991). The plasminogen activator family from the salivary gland of the vampire bat Desmodus rotundus: cloning and expression. Gene 105: 229–237.

Kruithof EK, Schleuning WD (2004). A comparative study of amyloid-beta (1-42) as a cofactor for plasminogen activation by vampire bat plasminogen activator and recombinant human tissue-type plasminogen activator. Thromb Haemost 92: 559–567.

Kwon M, MacLeod TJ, Zhang Y, Waisman DM (2005). S100A10, annexin A2, and annexin a2 heterotetramer as candidate plasminogen receptors. Front Biosci 10: 300–325.

Lees KR, Bluhmki E, von Kummer R, Brott TG, Toni D, Grotta JC *et al*. (2010). Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. Lancet 375: 1695–1703.

Liberatore GT, Samson A, Bladin C, Schleuning WD, Medcalf RL (2003). Vampire bat salivary plasminogen activator (desmoteplase): a unique fibrinolytic enzyme that does not promote neurodegeneration. Stroke 34: 537–543.

Liebeskind DS (2009). Reversing stroke in the 2010s: lessons from Desmoteplase in acute ischemic stroke-2 (DIAS-2). Stroke 40: 3156–3158.

Longstaff C, Thelwell C, Williams SC, Silva MM, Szabo L, Kolev K (2011). The interplay between tissue plasminogen activator domains and fibrin structures in the regulation of fibrinolysis: kinetic and microscopic studies. Blood 117: 661–668.

Lopez-Atalaya JP, Roussel BD, Ali C, Maubert E, Petersen KU, Berezowski V *et al*. (2007). Recombinant Desmodus rotundus salivary plasminogen activator crosses the blood-brain barrier through a low-density lipoprotein receptor-related protein-dependent mechanism without exerting neurotoxic effects. Stroke 38: 1036–1043.

Lopez-Atalaya JP, Roussel BD, Levrat D, Parcq J, Nicole O, Hommet Y *et al*. (2008). Toward safer thrombolytic agents in stroke: molecular requirements for NMDA receptor-mediated neurotoxicity. J Cereb Blood Flow Metab 28: 1212–1221.

Loscalzo J (1988). Structural and kinetic comparison of recombinant human single- and two-chain tissue plasminogen activator. J Clin Invest 82: 1391–1397.

Maas C, Schiks B, Strangi RD, Hackeng TM, Bouma BN, Gebbink MF *et al*. (2008). Identification of fibronectin type I domains as amyloid-binding modules on tissue-type plasminogen activator and three homologs. Amyloid 15: 166–180.

Madani R, Hulo S, Toni N, Madani H, Steimer T, Muller D *et al*. (1999). Enhanced hippocampal long-term potentiation and learning by increased neuronal expression of tissue-type plasminogen activator in transgenic mice. EMBO J 18: 3007–3012.

Mannaioni G, Orr AG, Hamill CE, Yuan H, Pedone KH, McCoy KL *et al*. (2008). Plasmin potentiates synaptic N-methyl-D-aspartate receptor function in hippocampal neurons through activation of protease-activated receptor-1. J Biol Chem 283: 20600–20611.

Matys T, Strickland S (2003). Tissue plasminogen activator and NMDA receptor cleavage. Nat Med 9: 371–372; author reply 372–373.

Medcalf RL, Ruegg M, Schleuning WD (1990). A DNA motif related to the cAMP-responsive element and an exon-located activator protein-2 binding site in the human tissue-type plasminogen activator gene promoter cooperate in basal expression and convey activation by phorbol ester and cAMP. J Biol Chem 265: 14618–14626.

Mellott MJ, Ramjit DR, Stabilito II, Hare TR, Senderak ET, Lynch JJ *et al.* (1995). Vampire bat salivary plasminogen activator evokes minimal bleeding relative to tissue-type plasminogen activator as assessed by a rabbit cuticle bleeding time model. Thromb Haemost 73: 478–483.

Montoney M, Gardell SJ, Marder VJ (1995). Comparison of the bleeding potential of vampire bat salivary plasminogen activator versus tissue plasminogen activator in an experimental rabbit model. Circulation 91: 1540–1544.

Mori T, Wang X, Kline AE, Siao CJ, Dixon CE, Tsirka SE *et al.* (2001). Reduced cortical injury and edema in tissue plasminogen activator knockout mice after brain trauma. Neuroreport 12: 4117–4120.

Muschick P, Zeggert D, Donner P, Witt W (1993). Thrombolytic properties of Desmodus (vampire bat) salivary plasminogen activator DSPA$\alpha_1$, alteplase and streptokinase following intravenous bolus injection in a rabbit model of carotid artery thrombosis. Fibrinolysis 7: 284–290.

Nicole O, Docagne F, Ali C, Margaill I, Carmeliet P, MacKenzie ET *et al.* (2001). The proteolytic activity of tissue-plasminogen activator enhances NMDA receptor-mediated signaling. Nat Med 7: 59–64.

Norris EH, Strickland S (2007). Modulation of NR2B-regulated contextual fear in the hippocampus by the tissue plasminogen activator system. Proc Natl Acad Sci USA 104: 13473–13478.

Orth K, Willnow T, Herz J, Gething MJ, Sambrook J (1994). Low density lipoprotein receptor-related protein is necessary for the internalization of both tissue-type plasminogen activator-inhibitor complexes and free tissue-type plasminogen activator. J Biol Chem 269: 21117–21122.

Pawlak R, Magarinos AM, Melchor J, McEwen B, Strickland S (2003). Tissue plasminogen activator in the amygdala is critical for stress-induced anxiety-like behavior. Nat Neurosci 6: 168–174.

Pawlak R, Melchor JP, Matys T, Skrzypiec AE, Strickland S (2005a). Ethanol-withdrawal seizures are controlled by tissue plasminogen activator via modulation of NR2B-containing NMDA receptors. Proc Natl Acad Sci USA 102: 443–448.

Pawlak R, Rao BS, Melchor JP, Chattarji S, McEwen B, Strickland S (2005b). Tissue plasminogen activator and plasminogen mediate stress-induced decline of neuronal and cognitive functions in the mouse hippocampus. Proc Natl Acad Sci USA 102: 18201–18206.

Qian Z, Gilbert ME, Colicos MA, Kandel ER, Kuhl D (1993). Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. Nature 361: 453–457.

Reddrop C, Moldrich RX, Beart PM, Farso M, Liberatore GT, Howells DW *et al.* (2005). Vampire bat salivary plasminogen activator (desmoteplase) inhibits tissue-type plasminogen activator-induced potentiation of excitotoxic injury. Stroke 36: 1241–1246.

Renatus M, Stubbs MT, Huber R, Bringmann P, Donner P, Schleuning WD *et al.* (1997). Catalytic domain structure of vampire bat plasminogen activator: a molecular paradigm for proteolysis without activation cleavage. Biochemistry 36: 13483–13493.

Rogove AD, Siao C, Keyt B, Strickland S, Tsirka SE (1999). Activation of microglia reveals a non-proteolytic cytokine function for tissue plasminogen activator in the central nervous system. J Cell Sci 112 (Pt 22): 4007–4016.

Sakharov DV, Barrertt-Bergshoeff M, Hekkenberg RT, Rijken DC (1999). Fibrin-specificity of a plasminogen activator affects the efficiency of fibrinolysis and responsiveness to ultrasound: comparison of nine plasminogen activators in vitro. Thromb Haemost 81: 605–612.

Samson AL, Medcalf RL (2006). Tissue-type plasminogen activator: a multifaceted modulator of neurotransmission and synaptic plasticity. Neuron 50: 673–678.

Samson AL, Nevin ST, Croucher D, Niego B, Daniel PB, Weiss TW *et al.* (2008). Tissue-type plasminogen activator requires a co-receptor to enhance NMDA receptor function. J Neurochem 107: 1091–1101.

Samson AL, Borg RJ, Niego B, Wong CH, Crack PJ, Yongqing T *et al.* (2009). A nonfibrin macromolecular cofactor for tPA-mediated plasmin generation following cellular injury. Blood 114: 1937–1946.

Sashindranath M, Samson AL, Downes CE, Crack PJ, Lawrence AJ, Li QX *et al.* (2011). Compartment- and context-specific changes in tissue-type plasminogen activator (tPA) activity following brain injury and pharmacological stimulation. Lab Invest (in press).

Schleuning WD (2001). Vampire bat plasminogen activator DSPA-alpha-1 (desmoteplase): a thrombolytic drug optimized by natural selection. Haemostasis 31: 118–122.

Schleuning WD, Donner P (2003). Desmodus rotundus (Vampire bat) plasminogen activator DSPA$\alpha_1$: a superior thrombolytic created by evolution. In: Sasahara AA, Loscazo J (eds). New Therepeutic Agents in Thrombosis and Thrombolysis. Marcel Dekker: New York, pp. 605–625.

Schleuning WD, Alagon A, Boidol W, Bringmann P, Petri T, Kratzschmar J *et al.* (1992). Plasminogen activators from the saliva of Desmodus rotundus (common vampire bat): unique fibrin specificity. Ann N Y Acad Sci 667: 395–403.

Seeds NW, Williams BL, Bickford PC (1995). Tissue plasminogen activator induction in Purkinje neurons after cerebellar motor learning. Science 270: 1992–1994.

Sipley JD, Alexander DS, Testa JE, Quigley JP (1997). Introduction of an RRHR motif into chicken urokinase-type plasminogen activator (ch-uPA) confers sensitivity to plasminogen activator inhibitor (PAI)-1 and PAI-2 and allows ch-uPA-mediated extracellular matrix degradation to be controlled by PAI-1. Proc Natl Acad Sci USA 94: 2933–2938.

Soehngen W, Petersen KU, Soehngen M (2010). Bat Plasminogen activator: Desmoteplase – from bat to bench to bedside of stroke victims. In: Kini RM (ed.). Toxins and Haemostasis. Springer Science+Business Media B.V.: Dordrecht, Heidelberg, London and New York, pp. 393–407.

Stubbs MT, Renatus M, Bode W (1998). An active zymogen: unravelling the mystery of tissue-type plasminogen activator. Biol Chem 379: 95–103.

Su EJ, Fredriksson L, Geyer M, Folestad E, Cale J, Andrae J *et al.* (2008). Activation of PDGF-CC by tissue plasminogen activator impairs blood-brain barrier integrity during ischemic stroke. Nat Med 14: 731–737.

Su EJ, Fredriksson L, Schielke GP, Eriksson U, Lawrence DA (2009). Tissue plasminogen activator-mediated PDGF signaling and neurovascular coupling in stroke. J Thromb Haemost 7 (Suppl. 1): 155–158.

Tellgren-Roth A, Dittmar K, Massey SE, Kemi C, Tellgren-Roth C, Savolainen P *et al.* (2009). Keeping the blood flowing-plasminogen activator genes and feeding behavior in vampire bats. Naturwissenschaften 96: 39–47.

Thelwell C, Longstaff C (2007). The regulation by fibrinogen and fibrin of tissue plasminogen activator kinetics and inhibition by plasminogen activator inhibitor 1. J Thromb Haemost 5: 804–811.

Tooyama I, Kawamata T, Akiyama H, Kimura H, Moestrup SK, Gliemann J *et al*. (1995). Subcellular localization of the low density lipoprotein receptor-related protein (alpha 2-macroglobulin receptor) in human brain. Brain Res 691: 235–238.

Tsirka SE, Gualandris A, Amaral DG, Strickland S (1995). Excitotoxin-induced neuronal degeneration and seizure are mediated by tissue plasminogen activator. Nature 377: 340–344.

Tsirka SE, Rogove AD, Strickland S (1996). Neuronal cell death and tPA. Nature 384: 123–124.

Van de Werf FJ (1999). The ideal fibrinolytic: can drug design improve clinical results? Eur Heart J 20: 1452–1458.

Wang YF, Tsirka SE, Strickland S, Stieg PE, Soriano SG, Lipton SA (1998). Tissue plasminogen activator (tPA) increases neuronal damage after focal cerebral ischemia in wild-type and tPA-deficient mice. Nat Med 4: 228–231.

Witt W, Baldus B, Bringmann P, Cashion L, Donner P, Schleuning WD (1992). Thrombolytic properties of Desmodus rotundus (vampire bat) salivary plasminogen activator in experimental pulmonary embolism in rats. Blood 79: 1213–1217.

Witt W, Maass B, Baldus B, Hildebrand M, Donner P, Schleuning WD (1994). Coronary thrombolysis with Desmodus salivary plasminogen activator in dogs. Fast and persistent recanalization by intravenous bolus administration. Circulation 90: 421–426.

Wolf BB, Lopes MB, VandenBerg SR, Gonias SL (1992). Characterization and immunohistochemical localization of alpha 2-macroglobulin receptor (low-density lipoprotein receptor-related protein) in human brain. Am J Pathol 141: 37–42.

Yepes M, Lawrence DA (2004). New functions for an old enzyme: nonhemostatic roles for tissue-type plasminogen activator in the central nervous system. Exp Biol Med (Maywood) 229: 1097–1104.

Zavalova LL, Basanova AV, Baskova IP (2002). Fibrinogen-fibrin system regulators from bloodsuckers. Biochemistry (Mosc) 67: 135–142.

van Zonneveld AJ, Veerman H, Pannekoek H (1986). On the interaction of the finger and the kringle-2 domain of tissue-type plasminogen activator with fibrin. Inhibition of kringle-2 binding to fibrin by epsilon-amino caproic acid. J Biol Chem 261: 14214–14218.